



Physiology

Selenium and sulfur influence ethylene formation and alleviate cadmium-induced oxidative stress by improving proline and glutathione production in wheat



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SUMMARY

We have studied the influence of selenium (Se) and sulfur (S) in the protection of photosynthetic capacity of wheat (*Triticum aestivum*) against cadmium (Cd) stress. The involvement of ethylene and its interaction with proline and antioxidant metabolism in the tolerance of plants to Cd stress was evaluated. Application of Se or S alleviated Cd-induced oxidative stress by increasing proline accumulation as a result of increased activity of glutamyl kinase (GK) and decreased activity of proline oxidase (PROX). These nutrients also induced the activity of ATP-sulfurylase and serine acetyl transferase and the content of cysteine (Cys), a precursor for the synthesis of both reduced glutathione (GSH) and ethylene. Further, application of Se and S to plants under Cd stress reduced ethylene level and increased the activity of glutathione reductase (GR) and glutathione peroxidase (GPX), reduced oxidative stress and improved photosynthesis and growth. The involvement of ethylene in Se and S-mediated alleviation of Cd stress was substantiated with the use of ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG). The use of AVG reversed the effects of Se and S on ethylene, content of proline and GSH and photosynthesis. The results suggested that Se and S both reversed Cd-induced oxidative stress by regulating ethylene formation, proline and GSH metabolism. Thus, Se or S-induced regulatory interaction between ethylene and proline and GSH metabolism may be used for the reversal of Cd-induced oxidative stress.

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Introduction

Cadmium (Cd) is a powerful pollutant due to its long half life in soil and greater solubility in water. Plants grown under excess Cd show reduced growth and metabolism. Elevated levels of Cd induce excessive production of reactive oxygen species (ROS). These ROS cause damage to photosynthetic apparatus resulting in adverse effects on photosynthetic potential of plants (Mobin and Khan,

2007; Astolfi et al., 2012; Dias et al., 2013; Asgher et al., 2014). Several efforts have been made to counteract Cd-induced toxicity and restore the photosynthetic ability of plants. Supplementation of plants with mineral elements is one of the strategies adopted. The added mineral elements benefit plants because of their known biological role in metabolism and also help to reduce the toxicity generated by Cd. It has been reported that inputs of essential or beneficial nutrients such as nitrogen (N), sulfur (S), iron (Fe) and selenium (Se) restore photosynthetic ability, improve antioxidants capacity and productivity of crop plants (Iqbal et al., 2011; Hasanuzzaman and Fujita, 2011; Hasanuzzaman et al., 2011; Astolfi et al., 2012; Asgher et al., 2014).

Sulfur is an essential mineral nutrient element and an integral part of certain amino acids (cysteine, Cys and methionine), antioxidant (reduced glutathione; GSH), co-enzymes, prosthetic groups, vitamins, secondary metabolites, phytochelatins (PCs) and lipids (Khan et al., 2014a,b). The role of S in detoxification of Cd-induced oxidative stress was correlated with adequate S availability in *Hordeum vulgare* and *Brassica juncea* plants with increased GSH production (Astolfi et al., 2012; Masood et al., 2012; Asgher et al., 2014).

Abbreviations: ACS, 1-aminocyclopropane carboxylic acid synthase; ATP-S, adenosine triphosphate sulfurylase; AVG, aminoethoxyvinylglycine; Cd, cadmium; Cys, cysteine; DAS, days after sowing; ETR, electron transport rate; GK, glutamyl kinase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; NPQ, non-photochemical quenching; NR, nitrate reductase; PROX, proline oxidase; PS, photosystem; qP, photochemical quenching; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; S, sulfur; SAT, serine acetyltransferase; Se, selenium; TBARS, thiobarbituric acid reactive substances.

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We have shown earlier that S application reduced the adverse effects of Cd and improved photosynthesis in *B. juncea* plants by enhancing the activity of antioxidant enzymes (Masood et al., 2012; Asgher et al., 2014).

Recent studies on Se have shown that this beneficial element also reduces the adverse effects of abiotic stress. The low concentration of Se stimulates growth and alleviates effects of Cd stress. While it promotes oxidant activity, it causes damages to plants at high concentration (Feng et al., 2013; Hawrylak-Nowak et al., 2014; Saidi et al., 2014). It has been reported that Se regulates ROS metabolism and induces activity of antioxidant enzymes (Djanaguiraman et al., 2010) resulting in reduced damage to growth of plants (Hasanuzzaman et al., 2011; Hawrylak-Nowak et al., 2014; Saidi et al., 2014). Recently, Hasanuzzaman et al. (2012) have shown that exogenous application of Se increases tolerance of *Brassica napus* plants to Cd-induced oxidative damage by enhancing the enzymatic and non-enzymatic antioxidant systems. Hawrylak-Nowak et al. (2014) have shown that Se reduced PCs accumulation in roots, but did not change its concentration in leaves of *Cucumis sativus* plants. Selenium has been found to increase GSH content in *B. napus* plants (Hasanuzzaman et al., 2012). In higher plants, Se metabolism is closely related to S because of its similar chemical properties. Selenium is considered a chemical analog of S and competes for the same transporters during uptake by roots (Mikkelsen and Wan, 1990; Zayed and Terry, 1992). Moreover, Cd up-regulates sulfate transporters (Sultr1;1 and Sultr2;1) (Takahashi et al., 2000; Herbette et al., 2006). Selenite resistant mutants of *Arabidopsis thaliana*, caused by a mutation in the high-affinity sulfate transporter Sultr1;2, showed decreased uptake of both Se and S (Shibagaki et al., 2002; El Kassis et al., 2007). Pilon-Smits et al. (1999) reported that Se and S were both involved in ATP-sulfurylase overexpression.

The roles of proline metabolism, GSH synthesis and ethylene formation have been identified in tolerance of plants to stress. The independent study on *Arabidopsis* (Yoshida et al., 2009) and *B. juncea* plants (Asgher et al., 2014) has shown that GSH production and ethylene formation are linked with ozone and Cd tolerance. Similarly, it has been shown that there exists an interaction between proline metabolism and ethylene formation for heat stress tolerance in *Triticum aestivum* (Khan et al., 2013). It is therefore, assumed that Se and S may modulate ethylene formation and proline and antioxidant metabolism for Cd tolerance. The inhibition of Cd-induced stress ethylene and oxidative stress by Se and S may result in the increase in sensitivity of plants to ethylene and promote proline and antioxidant metabolism. In the present study, we compared the effectiveness of Se and S in Cd stress tolerance and studied the tolerance induced through ethylene-mediated modulation of proline and GSH metabolism in *T. aestivum*.

Materials and methods

Plant material and growth conditions

Healthy seeds of wheat (*Triticum aestivum* L.) cv. WH 711 were surface sterilized with 95% ethyl alcohol followed by repeated washings with double distilled water. Seeds were sown in 23 cm diameter earthen pots filled with reconstituted soil (peat and compost, 4:1 (v/v); mixed with sand, 3:1 (v/v)). The treatments were 0 (control), 200 mg Cd kg⁻¹ soil, 2 mg Se kg⁻¹ soil, 200 mg S kg⁻¹ soil or Cd combined with Se or S. The sources for Cd, Se and S were cadmium chloride (CdCl₂), sodium selenite (Na₂SeO₃·5H₂O) and elemental sulfur, respectively. An amount of 326.2 mg CdCl₂ and 6.7 mg sodium selenite was taken for 200 mg Cd kg⁻¹ soil and 2 mg Se kg⁻¹ soil, respectively. Elemental sulfur was used to obtain 200 mg S kg⁻¹ soil. The required amounts for 2 mg Se kg⁻¹

soil and 200 mg S kg⁻¹ soil were mixed thoroughly with soil and left for 15 days. After 15 days the pots were filled with the soil and seed sowing was done. The Cd treatment was given at the time of seed sowing. The pots were kept in a naturally illuminated green house of the Botany Department, Aligarh Muslim University, Aligarh, India with day/night temperatures at 21 °C/17 °C (±3 °C), photosynthetically active radiation (PAR; 680 μmol m⁻² s⁻¹) and relative humidity of 68 ± 5%. Another experiment was conducted to substantiate the information that ethylene is involved in Se or S-induced Cd stress tolerance. For this, plants grown with Cd in the presence of Se or S were given foliar treatment of 50 μL L⁻¹ of aminoethoxyvinylglycine (AVG), an ethylene biosynthesis inhibitor. Earlier, independent experiments on Se and S have shown that 2 mg Se kg⁻¹ soil and 200 mg S kg⁻¹ soil have reduced oxidative stress (Chu et al., 2010; Masood et al., 2012). Treatments were arranged in a factorial randomized complete block design. The number of replicates for each treatment was four (n = 4). The measurements in the experiments were done at 30 DAS.

Determination of H₂O₂ content and lipid peroxidation

The content of H₂O₂ was determined following the method of Okuda et al. (1991). Fresh leaf tissues (500 mg) were ground in ice-cold 200 mM perchloric acid. After centrifugation at 1200 × g for 10 min, perchloric acid of supernatant was neutralized with 4 M KOH. Insoluble potassium perchlorate was eliminated by centrifugation at 500 × g for 3 min. In a final volume of 1.5 mL, 1 mL of the eluate, 400 μL of 12.5 mM 3-(dimethylamino) benzoic acid (DMAB) in 0.375 M phosphate buffer (pH 6.5), 80 μL of 3-methyl-2-benzothiazoline hydrazone (MBTH) and 20 μL of peroxidase (0.25 U) were added. The reaction was started by the addition of peroxidase at 25 °C and the increase in absorbance was recorded at 590 nm on a UV–Vis spectrophotometer.

Lipid peroxidation in leaves was determined by estimating the content of TBARS as described by Dhindsa et al. (1981). Fresh leaf tissues (500 mg) were ground in 0.25% 2-thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using mortar and pestle. After heating at 95 °C for 30 min, the mixture was rapidly cooled on ice bath and centrifuged at 10,000 × g for 10 min. To 1 mL aliquot of the supernatant 4 mL 20% TCA containing 0.5% TBA was added. The absorbance of the supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance of the same at 600 nm. The content of TBARS was calculated using the extinction coefficient (155 mM⁻¹ cm⁻¹).

Measurements of gas exchange parameters, Rubisco activity, chlorophyll and growth

Gas exchange parameters (net photosynthesis, stomatal conductance and intercellular CO₂ concentration) were measured in fully expanded uppermost leaves of plants using infrared gas analyzer (CID-340, Photosynthesis System, Bio-Science, USA). The measurements were done on a sunny day at light saturating intensity; PAR; 720 μmol m⁻² s⁻¹ and at 370 ± 5 μmol mol⁻¹ atmospheric CO₂ concentrations. Chlorophyll was measured in intact leaves with the help of SPAD chlorophyll meter (502 DL PLUS, Spectrum Technologies, USA).

The activity of ribulose 1,5-bisphosphate carboxylase (Rubisco; EC 4.1.1.39) was determined adopting the method of Usuda (1985) by monitoring NADH oxidation at 30 °C at 340 nm during the conversion of 3-phosphoglycerate to glycerol 3-phosphate after addition of enzyme extract to the assay medium. For enzyme extraction, leaf tissue (1.0 g) was homogenized using a chilled mortar and pestle with ice-cold extraction buffer containing 0.25 M Tris–HCl (pH 7.8), 0.05 M MgCl₂, 0.0025 M EDTA and

37.5 mg dithiothreitol (DTT). This homogenate was centrifuged at 4 °C at $10,000 \times g$ for 10 min. The resulting supernatant was used to assay the enzyme. The reaction mixture (3 mL) contained 100 mM Tris–HCl (pH 8.0), 40 mM NaHCO_3 , 10 mM MgCl_2 , 0.2 mM NADH, 4 mM ATP, 5 mM DTT, 1U of glyceraldehyde 3-phosphodehydrogenase, 1U of 3-phosphoglycerate kinase and 0.2 mM ribulose 1,5-bisphosphate (RuBP).

Plants were uprooted carefully from the pots and washed to remove dust. Leaf area was measured using a leaf area meter (LA 211, Systronics, New Delhi, India). Dry mass of plants was recorded after drying the sample in a hot air oven at 80 °C till constant weight.

Measurement of chlorophyll fluorescence parameters

Fully expanded leaves were allowed to adapt under dark condition for 30 min before chlorophyll fluorescence measurements using Junior-PAM chlorophyll fluorometer (Heinz Walz, Germany). Minimal fluorescence (F_0) and maximum fluorescence (F_m) were measured in dark-adapted leaves with a low measuring beam at a light intensity of $125 \mu\text{mol m}^{-2} \text{s}^{-1}$, whereas under light-adapted condition, minimal fluorescence (F_0') and maximum fluorescence (F_m') were measured in the same leaves with a saturating light intensity ($720 \mu\text{mol m}^{-2} \text{s}^{-1}$) together with steady-state fluorescence (F_s). The variable fluorescence (F_v and F_v') was calculated using the values of $F_m - F_0$ and $F_m' - F_0'$, and actual PSII efficiency (Φ PS II) was determined as $F_m' - F_s / F_m'$, maximal efficiency of PS II by using F_v / F_m and intrinsic efficiency of PS II by using F_v' / F_m' . Using fluorescence parameters determined in both the light- and dark-adapted states, the photochemical quenching (qP) and non-photochemical quenching (NPQ) parameters were calculated. Photochemical quenching was calculated as $(F_m' - F_s) / F_v'$ and NPQ as $(F_m - F_m') / F_m'$ (Maxwell and Johnson, 2000). Electron transport rate (ETR) was calculated by the following formula: Φ PS II \times photosynthetic photon flux density $\times 0.5 \times 0.84$ as suggested by Krall and Edwards (1992).

Estimation of proline

Proline content was determined spectrophotometrically by adopting the ninhydrin method of Bates et al. (1973). Fresh leaf samples (300 mg) were homogenized in 3 mL of 3% sulphosalicylic acid. The homogenate filtrate was reacted with 1 mL each of acid ninhydrin and glacial acetic acid for 1 h in a test tube placed in a water bath at 100 °C. The mixture was extracted with toluene and absorbance was measured at 520 nm using L-proline as a standard.

Activity of glutamyl kinase and proline oxidase

To determine the activity of glutamyl kinase (GK; EC 2.7.2.11) and proline oxidase (PROX; EC 1.5.99.8), enzyme extract was prepared by homogenizing 500 mg leaf sample in 0.1 M Tris–HCl buffer (pH 7.5) at 4 °C. The homogenate was centrifuged at $30,000 \times g$ for 30 min, the pellet collected and used as extract for assay of GK and PROX. For GK enzyme activity extract was kept in a freezer at –20 °C. Activity of GK was assayed by the method of Hayzer and Leisinger (1980) with a slight modification. The frozen sample was suspended in 10 mL of 0.1 M Tris–HCl buffer containing 1 mM DTT to rupture the cell and was centrifuged at $30,000 \times g$ for 30 min. The assay mixture contained 50 mM L-glutamate, 10 mM ATP, 20 mM MgCl_2 , 100 mM hydroxylamine HCl and 50 mM Tris–HCl (pH 7.0) with 200 μL of desalted extract in a final volume of 500 μL . The reaction was initiated by addition of enzyme extract. After 30 min of incubation at 37 °C, the reaction was stopped by the addition of 1.0 mL $\text{FeCl}_3 \cdot 3\text{H}_2\text{O}$ (2.5%, w/v) and trichloroacetic acid (6%, w/v) in 2.5 M HCl. Protein was precipitated and removed by centrifugation at $12,000 \times g$ at 4 °C and absorbance was recorded at 540 nm.

Activity of glutamyl kinase was expressed in U mg^{-1} protein. One unit (U) of the enzyme activity is defined as μg of glutamyl hydroxamate produced $\text{min}^{-1} \text{mg}^{-1}$ protein.

The activity of PROX was determined adopting the method of Huang and Cavalieri (1979) with a slight modification. The pellet was mixed with 1 mL Tricine, KOH buffer (pH 7.5) containing 6 M sucrose. This extract was used for the enzyme assay. The assay mixture contained 1.2 mL of 50 mM Tris–HCl buffer (pH 8.5), 1.2 mL of 5 mM MgCl_2 , 0.1 mL of 0.5 mM NADP, 0.1 mL of 1 mM KCN, 0.1 mL of 1 mM phenazine methosulfate (PSM), 0.1 mL of 0.06 mM 2, 6 dichlorophenol indophenol (DCPIP) and 0.1 mL of 0.1 M proline in a final volume of 3 mL. Increase in absorbance was recorded at 600 nm at 25 °C using proline to initiate the reaction. Proline oxidase activity was expressed in U mg^{-1} protein. One unit (U) of enzyme activity is defined as mM DCPIP reduced $\text{min}^{-1} \text{mg}^{-1}$ protein.

Determination of nitrate reductase activity and leaf nitrogen content

Activity of nitrate reductase (NR; EC 1.7.99.4) in leaf was measured by preparing enzyme extract using the method of Kuo et al. (1982). Leaves (1.0 g) were frozen in liquid N_2 , ground to a powder with mortar and pestle, and then stored at –80 °C. The powder was thawed for 10 min at 4 °C and homogenized in a blender in 250 mM Tris–HCl buffer (pH 8.5), containing 10 mM cysteine, 1 mM EDTA, 20 M FAD, 1 mM DTT, and 10% (v/v) glycerol. The homogenate was centrifuged at $10,000 \times g$ for 30 min at 4 °C. Activity of NR was assayed spectrophotometrically as the rate of nitrite production at 28 °C adopting the procedure of Nakagawa et al. (1984). The assay mixture contained KNO_3 (10 mM), HEPES (0.065 M; pH 7.0), NADH (0.5 mM) in phosphate buffer (0.04 mM; pH 7.2) and the enzyme in a final volume of 1.5 mL. The reaction was started by adding NADH. After 15 min, the reaction was terminated by adding 1 mL of 1 N HCl solution containing 1% sulfanilamide followed by the addition of 1 mL of 0.02% aqueous N-1-naphthylethylene-di-amine-dihydrochloride. The absorbance was read at 540 nm after 10 min.

Leaf N content was determined in acid-peroxide digested material using the method of Lindner (1944).

Activity of ATP-sulfurylase and serine acetyl transferase

Activity of ATP-sulfurylase (ATP-S; EC 4.4.1.14) was measured using molybdate-dependent formation of pyrophosphate as described by Lappartient and Touraine (1996). Fresh leaf tissue (1.0 g) was ground at 4 °C in a buffer consisting of 10 mM Na_2EDTA , 20 mM Tris–HCl (pH 8.0), 2 mM DTT, and 0.01 g mL^{-1} PVP, using 1:4 (w/v) tissue to buffer ratio. The homogenate was centrifuged at $20,000 \times g$ for 10 min at 4 °C. The supernatant was used for *in vitro* ATP-sulfurylase assay. The reaction was initiated by adding 0.1 mL of extract to 0.5 mL of reaction mixture, which contained 7 mM MgCl_2 , 5 mM Na_2MoO_4 , 2 mM Na_2ATP , and 0.032 U mL^{-1} of sulfate-free inorganic pyrophosphate in 80 mM Tris–HCl buffer (pH 8.0). Another aliquot from the same extract was added to the same reaction mixture but without Na_2MoO_4 . Incubations were carried out at 37 °C for 15 min, after which phosphate was determined using spectrophotometer.

Activity of serine acetyl transferase (SAT; EC 2.3.1.30) in the leaf extract was determined by the method of Kredich and Tomkins (1966). Fresh leaf tissues (0.5 g) were ground with a chilled mortar and pestle in 2 mL of ice cold extraction buffer Tris–HCl 100 mM (pH 8.0), 100 mM KCl, 20 mM MgCl_2 , 1% Tween 80, and 10 mM DTT. The samples were transferred to microcentrifuge tubes and spun at 11, 600 $\times g$ for 10 min at 4 °C. The supernatant obtained was used for the SAT assay. The enzyme reaction mixture contained

Table 1

Content of H₂O₂ and TBARS, net photosynthesis, stomatal conductance, intercellular CO₂ concentration, Rubisco activity, chlorophyll content, leaf area and dry mass of wheat plants. Plants were grown individually with 0, 200 mg Cd kg⁻¹ soil, 2 mg Se kg⁻¹ soil and 200 mg S kg⁻¹ soil or Cd combined with Se or S. Data are presented as treatments mean \pm SE ($n = 4$).

	Control	Cd	Se	S	Se + Cd	S + Cd
H ₂ O ₂ content (nmol g ⁻¹ FW)	50.7 \pm 3.4c	145.1 \pm 8.9a	27.5 \pm 2.8d	28.2 \pm 2.6d	68.4 \pm 3.8b	69.0 \pm 3.9b
TBARS content (nmol g ⁻¹ FW)	18.3 \pm 1.6c	40.7 \pm 3.1a	11.8 \pm 1.1d	11.4 \pm 1.2d	24.2 \pm 2.5b	24.9 \pm 2.3b
Net photosynthesis (μ mol CO ₂ m ⁻² s ⁻¹)	10.9 \pm 0.89c	7.6 \pm 0.47d	18.2 \pm 1.56a	18.4 \pm 1.43a	13.2 \pm 1.12b	13.5 \pm 1.12b
Stomatal conductance (mmol CO ₂ m ⁻² s ⁻¹)	397 \pm 28.7c	289 \pm 15.3d	546 \pm 30.2a	548 \pm 33.7a	484 \pm 26.7b	489 \pm 26.8b
Intercellular CO ₂ (μ mol CO ₂ mol ⁻¹)	249 \pm 14.7c	167 \pm 7.6d	363 \pm 16.3a	365 \pm 23.2a	295 \pm 19.6b	299 \pm 22.4b
Rubisco activity (μ mol CO ₂ m ⁻² s ⁻¹)	41.3 \pm 1.52c	29.7 \pm 1.24d	53.5 \pm 1.86a	53.9 \pm 1.79a	49.2 \pm 1.65b	49.6 \pm 1.70b
Chlorophyll content (SPAD value)	31.7 \pm 1.61c	20.1 \pm 1.08d	43.3 \pm 1.58a	44.0 \pm 1.53a	37.5 \pm 1.39b	36.9 \pm 1.42b
Leaf area (cm ² plant ⁻¹)	29.6 \pm 1.43c	22.7 \pm 1.22d	58.8 \pm 1.92a	59.3 \pm 3.14a	49.8 \pm 1.95b	49.5 \pm 2.91b
Plant dry mass (g plant ⁻¹)	0.97 \pm 0.05c	0.69 \pm 0.03d	1.90 \pm 0.09a	1.89 \pm 0.09a	1.59 \pm 0.07b	1.56 \pm 0.08b

Data followed by same letter are not significantly different by LSD test at ($P < 0.05$).

0.1 mM acetyl CoA, 50 mM Tris-HCl (pH 7.6), 1 mM DTNB, 1 mM EDTA, and 1 mM L-serine in 1 mL. Subsequent to reaction initiation by addition of enzyme at 25 °C, initial rate was estimated by monitoring the increase in absorbance at 412 nm and calculated using an extinction coefficient of 13, 600 for thionitrobenzoic acid. A blank containing all materials except L-serine was run simultaneously and subtracted from the reaction rate obtained with L-serine.

Determination of content of sulfur, cysteine and GSH

Sulfur content was determined in leaf samples digested in a mixture of concentrated HNO₃ and 60% HClO₄ (85:1 v/v) using turbidimetric method of [Chesnin and Yien \(1950\)](#).

Cysteine content in leaves was determined spectrophotometrically adopting the method of [Giatonde \(1967\)](#). Fresh leaf (500 mg) tissue was homogenized in 5% (w:v) ice-cold perchloric acid. The suspension was centrifuged at 2800 \times g for 1 h at 5 °C and the supernatant was filtered. One mL of filtrate was treated with acid ninhydrin reagent and the absorption was read at 580 nm. The amount of Cys was calculated using calibration curve obtained for standard Cys.

Glutathione content was determined following the method of [Anderson \(1985\)](#). Fresh leaves (500 mg) were homogenized in 2.0 mL of 5% sulphosalicylic acid under cold conditions. The homogenate was centrifuged at 10,000 \times g for 10 min. To 0.5 mL of supernatant, 0.6 mL of phosphate buffer (100 mM, pH 7.0) and 40 μ L of 5'5'-dithiobis-2-nitrobenzoic acid (DTNB) were added. After 2 min the absorbance was read at 412 nm.

Assay of antioxidant enzymes

Fresh leaf tissue (200 mg) was homogenized with an extraction buffer containing 0.05% (v/v) Triton X-100 and 1% (w/v) PVP in potassium-phosphate buffer (100 mM, pH 7.0) using chilled mortar and pestle. The homogenate was centrifuged at 15,000 \times g for 20 min at 4 °C, and the supernatant obtained after centrifugation was used for assay of GSH reductase (GR; EC 1.6.4.2) and GSH peroxidase (GPX; EC 1.11.1.9) enzymes.

Activity of GR was determined adopting the method of [Foyer and Halliwell \(1976\)](#) by monitoring the GSH-dependent oxidation of NADPH. The assay mixture contained phosphate buffer (25 mM, pH 7.8), 0.5 mM oxidized GSH, 0.2 mM NADPH, and the enzyme extract. Activity of GR was calculated by using extinction coefficient

at 6.2 mM⁻¹ cm⁻¹. One Unit of the enzyme was amount necessary to decompose 1 μ mol of NADPH min⁻¹ at 25 °C.

Activity of GPX was determined by modified method of [Hasanuzzaman et al. \(2012\)](#). One mL reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM Na₂S₂O₈, 0.2 mM NADPH, 1 U mL⁻¹ GR, 1 mM GSH and 0.25 mM H₂O₂.

Oxidation of NADPH was recorded at 340 nm and activity of the enzymes was calculated using extinction coefficient at 6.2 mM⁻¹ cm⁻¹. One unit of the enzyme was amount necessary to decompose 1 μ mol of NADPH min⁻¹ at 25 °C.

Protein was estimated according to [Bradford \(1976\)](#) using bovine serum albumin as standard.

Measurement of ACS activity and ethylene production

Activity of ACS (EC 4.4.1.14) was measured by adopting the methods of [Avni et al. \(1994\)](#) and [Woeste et al. \(1999\)](#). Leaf tissue (5.0 g) was ground in 100 mM HEPES buffer (pH 8.0) containing 4 mM DTT, 2.5 mM pyridoxal phosphate and 25% PVP. The homogenized preparation was centrifuged at 12,000 \times g for 15 min. One mL of supernatant was placed in a 30 mL tube and 0.1 mL of 5 mM S-adenosyl methionine (AdoMet) was added and incubated for 2 h at 22 °C. The ACC formed was determined by its conversion to ethylene by addition of 0.1 mL of 20 mM HgCl₂ followed by the addition of 0.1 mL of a 1:1 mixture of saturated NaOH/NaCl and placed on ice for 10 min. In the control set, AdoMet was not added.

Ethylene was measured by cutting 0.5 g of leaf material into small pieces. These leaf pieces were placed into 30 mL tubes containing moist paper to minimize evaporation from the tissue, stoppered with secure rubber caps and placed in light for 2 h under the same condition used for plant growth. An earlier experiment showed that 2 h incubation time was adequate for ethylene detection without the interference of wound-induced ethylene, which began after 2 h of leaf incubation. One mL gas sample from the tubes was withdrawn with a hypodermic syringe and assayed on a gas chromatograph (Nucon 5700, New Delhi, India) fitted with 1.8 m Porapack N (80–100 mesh) column, a flame ionization detector and data station. Nitrogen was used as the carrier gas. The flow rates of nitrogen, hydrogen and oxygen were 30, 30 and 300 mL min⁻¹, respectively. The detector was set at 150 °C. Ethylene was identified based on the retention time and quantified by comparison with peaks from standard ethylene concentration.

Statistical analysis

Data were analyzed statistically using analysis of variance (ANOVA) by SPSS 17.0 for Windows, and presented as treatment mean \pm SE ($n=4$). Least significant difference (LSD) was calculated for significant data at $P<0.05$. Data followed by same letter are not significantly different by LSD test at $P<0.05$.

Results

Application of Se and S reduces H_2O_2 content and lipid peroxidation under Cd stress

Cadmium grown plants showed greater oxidative stress by exhibiting enhanced H_2O_2 content and lipid peroxidation by about 3-times and 2.5-times, respectively compared to control plants. To assess the influence of Se and S in reducing the Cd-induced oxidative stress, H_2O_2 content and lipid peroxidation were analyzed after application of Se and S. It proved effective in lowering oxidative stress under Cd stress. Treatments with Se and S reduced H_2O_2 content and lipid peroxidation equally by about 52.5% and 38.8%, respectively in Cd-treated plants compared with Cd-grown plants (Table 1).

Influence of Se and S on Rubisco activity, chlorophyll, gas exchange parameters and growth

Cadmium treatment decreased net photosynthesis, stomatal conductance and Rubisco activity by about 27%, and intercellular CO_2 concentration and chlorophyll by 30% compared to control plants. Application of Se and S improved gas exchange parameters in the absence of Cd and alleviated Cd-induced photosynthetic inhibition compared to control plants. Selenium and S under Cd stress increased net photosynthesis, stomatal conductance, internal CO_2 concentration and Rubisco activity equally by about 20% and chlorophyll by 18% compared to control plants (Table 1). Application of Se and S increased growth of plants grown without Cd, and ameliorated decrease in leaf area and plant dry mass with Cd compared to control (Table 1).

Influence of Se and S on chlorophyll fluorescence parameters under Cd stress

Chlorophyll fluorescence parameters were measured both under Cd stress and no stress and with or without treatments with Se and S. Cadmium treatment reduced the studied fluorescence parameters ($\Phi PS II$, F_v/F_m , F_v'/F_m' , ETR, and qP) compared to control, but treatment with Se and S increased these parameters. In contrast, NPQ increased with Cd treatment and significantly decreased with Se and S supplementation under no stress compared to control. Follow-up treatment with Se and S to Cd-treated plants proved effective in improving $\Phi PS II$, F_v/F_m , F_v'/F_m' , ETR, and qP compared to control plants (Table 2).

Table 2

Actual PS II efficiency, maximum PS II efficiency, intrinsic PS II efficiency, photochemical quenching, non-photochemical quenching and electron transport rate of wheat plants. Plants were grown individually with 0, 200 mg Cd kg⁻¹ soil, 2 mg Se kg⁻¹ soil and 200 mg S kg⁻¹ soil or Cd combined with Se or S. Data are presented as treatments mean \pm SE ($n=4$).

	Control	Cd	Se	S	Se + Cd	S + Cd
Actual PS II efficiency ($\Phi PS II$)	0.619 \pm 0.053c	0.493 \pm 0.037d	0.756 \pm 0.061a	0.761 \pm 0.058a	0.707 \pm 0.045b	0.710 \pm 0.042b
Maximum PS II efficiency (F_v/F_m)	0.825 \pm 0.071c	0.679 \pm 0.062d	0.871 \pm 0.079a	0.876 \pm 0.074a	0.857 \pm 0.076b	0.859 \pm 0.078b
Intrinsic PS II efficiency (F_v'/F_m')	0.721 \pm 0.067c	0.624 \pm 0.053d	0.808 \pm 0.076a	0.810 \pm 0.074a	0.759 \pm 0.068b	0.760 \pm 0.063b
Photochemical quenching (qP)	0.849 \pm 0.079c	0.782 \pm 0.070d	0.907 \pm 0.084a	0.909 \pm 0.085a	0.875 \pm 0.079b	0.877 \pm 0.074b
Non-photochemical quenching (NPQ)	0.688 \pm 0.064c	0.910 \pm 0.085a	0.539 \pm 0.043d	0.541 \pm 0.041d	0.719 \pm 0.040b	0.721 \pm 0.040b
Electron transport rate (ETR)	187.2 \pm 8.60d	149.1 \pm 7.50e	229.6 \pm 10.67b	239.7 \pm 10.60a	213.8 \pm 9.40c	214.7 \pm 9.30c

Data followed by same letter are not significantly different by LSD test at ($P<0.05$).

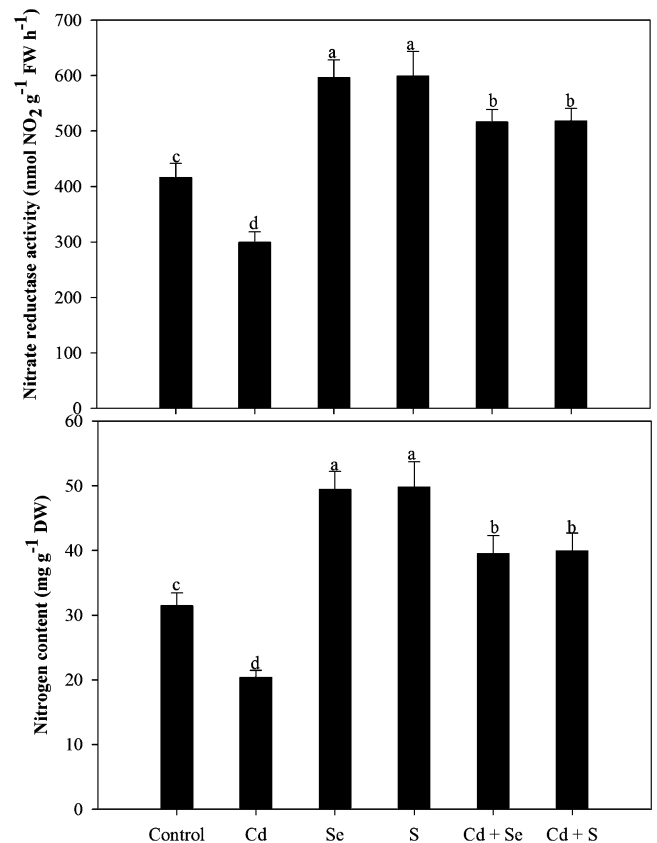


Fig. 1. Nitrate reductase activity and nitrogen content in wheat at 30 DAS. Plants were grown individually with 0, 200 mg Cd kg⁻¹ soil, 2 mg Se kg⁻¹ soil and 200 mg S kg⁻¹ soil or Cd combined with Se or S. Data are presented as treatments mean \pm SE ($n=4$). Data followed by same letter are not significantly different by LSD test at ($P<0.05$).

Influence of Se and S on N assimilation and proline metabolism under Cd stress

Cadmium stress decreased NR activity and N content by 28.5% and 35.2%, respectively compared to control plants. Application of Se and S resulted in increase in NR activity and N content equally by 43.9% and 58.2% under no stress and 24.5% and 26.9% under Cd stress compared to control plants (Fig. 1). To assess the role of proline metabolism in Cd tolerance under the influence of Se and S, the activity of proline metabolizing enzymes, GK and PROX were studied under Cd stress. Proline accumulation increased equally with Se and S treatments under no stress and Cd stress. Cadmium stress increased proline content by 45% in comparison to control. Application of Se and S increased proline accumulation under Cd stress and increased the content about 2.5-times under Cd stress compared to control (Fig. 2).

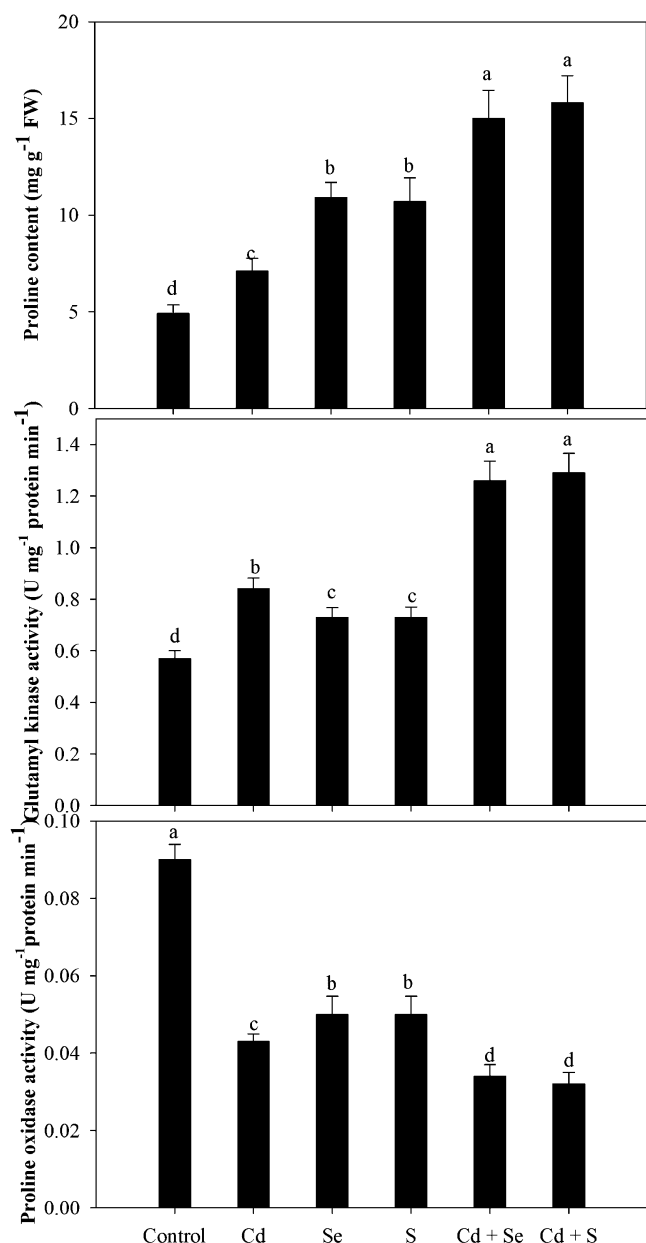


Fig. 2. Proline content, glutamyl kinase activity and proline oxidase activity in wheat at 30 DAS. Plants were grown individually with 0, 200 mg Cd kg⁻¹ soil, 2 mg Se kg⁻¹ soil and 200 mg S kg⁻¹ soil or Cd combined with Se or S. Data are presented as treatments mean \pm SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at ($P < 0.05$).

Treatment with Cd enhanced the activity of GK by 32.2% compared to control plants. Treatments with Se and S under Cd stress increased GK activity equally by about 1.3-times compared to control plants. But activity of PROX reduced under no stress and Cd-stressed plants with Se and S treatments. Application of Se and S reduced PROX activity by 1.8-times in Cd-stressed plants compared to control plants (Fig. 2).

Influence of Se and S on ATP-S, SAT, GR and GPX activity and content of S, cysteine and GSH under Cd stress

The activity of ATP-S, SAT, GR and GPX increased in plants receiving Se and S treatments and no stress compared to control plants. Treatment with Cd increased ATP-S, SAT, GR and GPX activity by 24.3%, 12.1%, 23.1% and 40.0%, respectively compared to control.

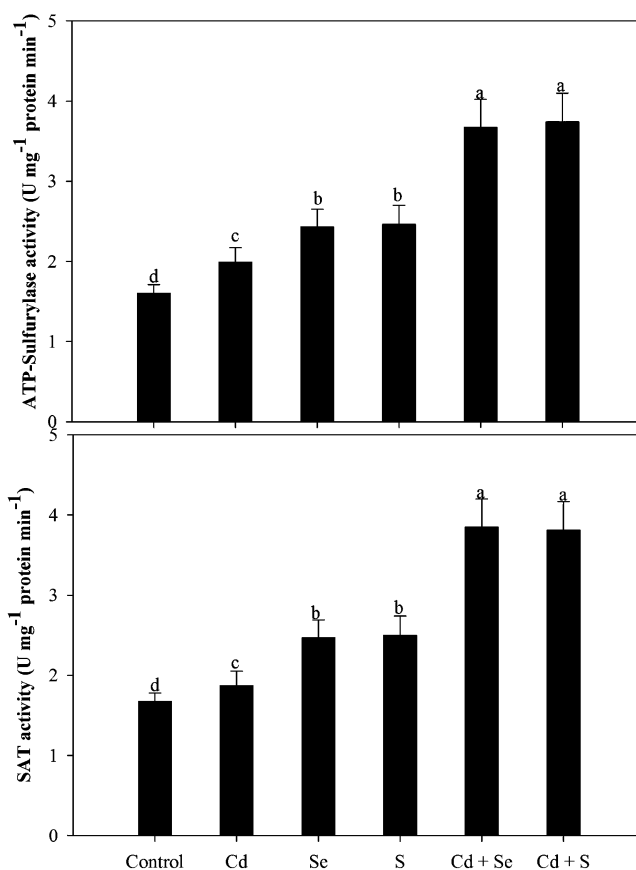


Fig. 3. ATP-sulfurylase activity and SAT activity in wheat at 30 DAS. Plants were grown individually with 0, 200 mg Cd kg⁻¹ soil, 2 mg Se kg⁻¹ soil and 200 mg S kg⁻¹ soil or Cd combined with Se or S. Data are presented as treatments mean \pm SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at ($P < 0.05$).

The application of Se and S under Cd stress increased the activity of ATP-S and SAT equally by 1.3-times, and GR and GPX by about 90% compared to control (Figs. 3 and 4). In contrast, S content decreased with Cd, but content of Cys and GSH increased in comparison to control. Selenium and S application increased the content of S by 71%, Cys by 60% and GSH by 18% compared to control under no stress condition. Selenium and S treatments to Cd-stressed plants reversed the adverse effect of Cd on S content and increased the content by 18%, while Cys and GSH contents were increased by 82% and 43% compared to control (Fig. 5).

Influence of Se and S on ACS activity and ethylene production under Cd stress

To assess the role of ethylene in Cd stress tolerance under the influence of Se and S, ACS activity and ethylene production were measured. Cadmium stress increased ACS activity by about 1.5-times and ethylene production by about 2-times compared to control. The plants treated with Se and S showed lesser ACS activity and ethylene production compared to control. Under Cd stress, supplementation of Se and S reduced the ACS activity and ethylene production equally compared to Cd-treated plants (Fig. 6).

Inhibiting ethylene biosynthesis using AVG reversed the effects of Se and S on Cd-induced proline metabolism, GSH production and net photosynthesis

From the results demonstrated above it is apparent that ethylene plays a role in Se and S-mediated reversal of Cd stress through reduction of ethylene production under Cd stress. To substantiate the role of ethylene in Se and S-induced Cd stress tolerance,

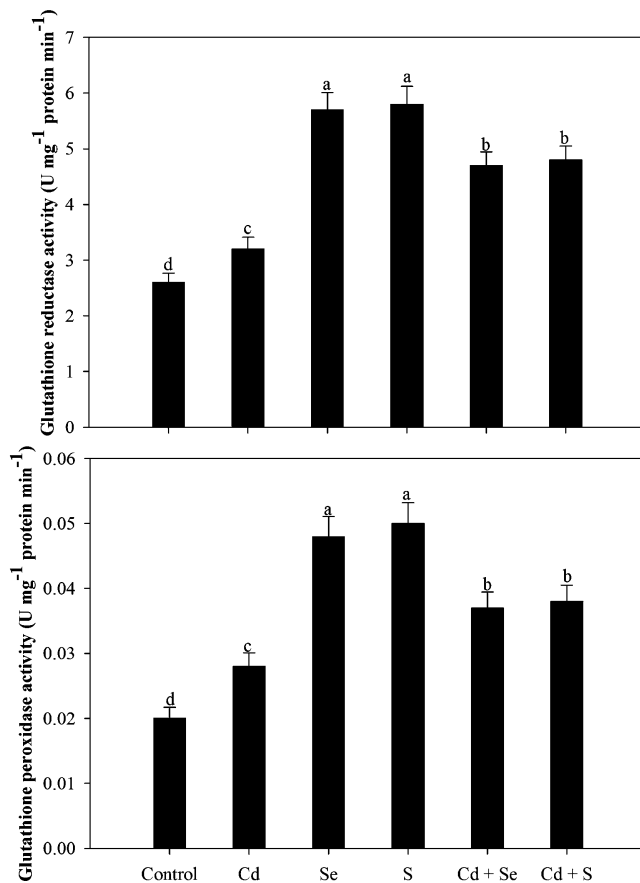


Fig. 4. GR activity and GPX activity in wheat at 30 DAS. Plants were grown individually with 0, 200 mg Cd kg⁻¹ soil, 2 mg Se kg⁻¹ soil and 200 mg S kg⁻¹ soil or Cd combined with Se or S. Data are presented as treatments mean \pm SE ($n=4$). Data followed by same letter are not significantly different by LSD test at ($P<0.05$).

ethylene biosynthesis was inhibited using AVG and content of GSH and proline and net photosynthesis were measured. The inhibition of ethylene in plants grown with Cd and supplemented with Se and S resulted in reduced proline and GSH content and inhibition of photosynthesis (Figs. 7 and 8).

Discussion

The contamination of agricultural soils by excess Cd has become a serious threat to the productivity of crop plants. Cadmium inhibits essential plant nutrients uptake, homeostatic balance and development of crop plants (Nazar et al., 2012). Selenium and S both are naturally occurring elements with similar chemical properties (White et al., 2004). Sulfur is an essential element with diverse metabolic functions (Khan et al., 2014a,b), whereas Se is a beneficial element (White et al., 2004). It has been found that Se takes up the function of S and benefits the development of plants because of its similar chemical properties with S (Mikkelsen and Wan, 1990; Zayed and Terry, 1992). It has been shown that S alleviates Cd stress through its involvement in ethylene and antioxidant metabolism (Masood et al., 2012). Selenium has also shown to reverse the effects of salt stress (Hasanuzzaman et al., 2011), cold stress (Chu et al., 2010), water stress (Soleimanzadeh, 2012) and Cd stress (Hawrylak-Nowak et al., 2014; Saidi et al., 2014). However, the influence of Se on ethylene production and antioxidant metabolism has not been reported. The reported research was undertaken to study the comparative effect of Se and S in the alleviation of Cd stress. The role of ethylene, proline and antioxidant metabolism in Se and S-mediated alleviation of Cd stress was also assessed.

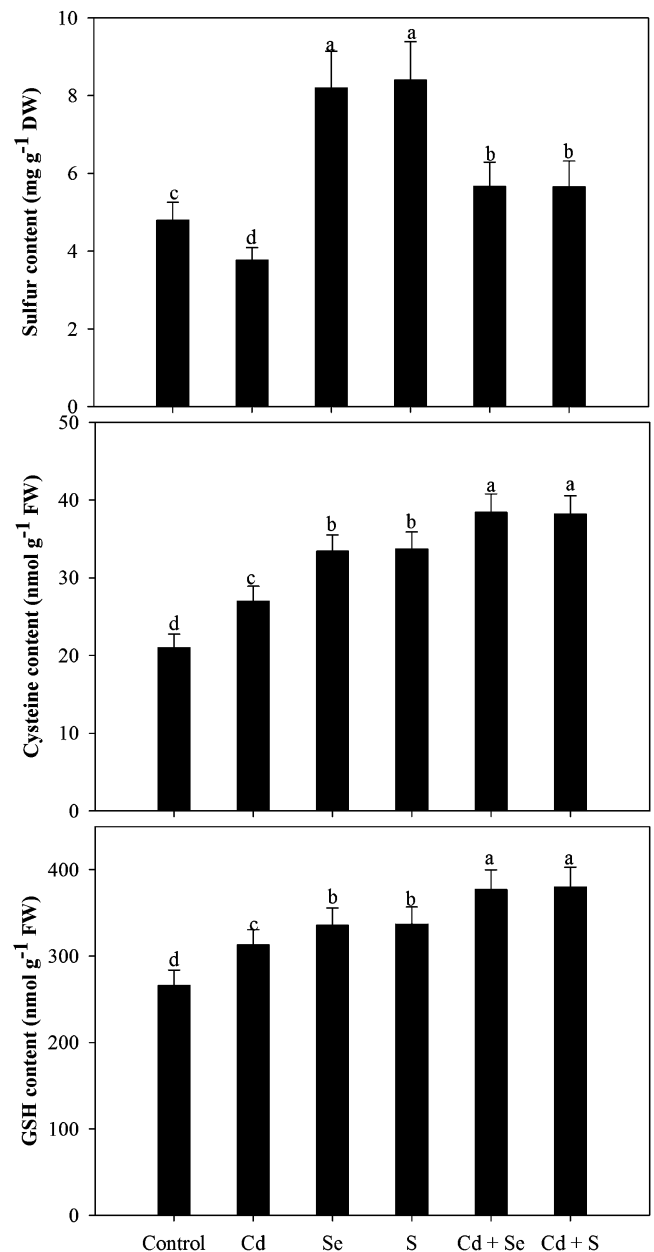


Fig. 5. Content of sulfur, cysteine and GSH in wheat at 30 DAS. Plants were grown individually with 0, 200 mg Cd kg⁻¹ soil, 2 mg Se kg⁻¹ soil and 200 mg S kg⁻¹ soil or Cd combined with Se or S. Data are presented as treatments mean \pm SE ($n=4$). Data followed by same letter are not significantly different by LSD test at ($P<0.05$).

Cadmium stress adversely affects N assimilation and photosynthetic attributes (Chaffei et al., 2004). In the present study, the decreased NR activity and N content under Cd stress were restored with the application of Se and S. This accounted for higher allocation of N to Rubisco protein leading to increased photosynthesis and growth of *T. aestivum* plants under Cd stress. Recently, Khan et al. (2013) have shown that increased N assimilation in plants increased proline synthesis, and involvement of ethylene in regulation of proline synthesis and photosynthesis under heat stress was observed. The present study has shown that Se and S increased proline metabolism and alleviated Cd-induced oxidative stress as evidenced by lower values of H₂O₂ content and lipid peroxidation. Proline accumulation is an adaptive strategy of plants to stressful environment which maintains the osmotic balance, scavenges excess free radicals, stabilizes cell membrane structure

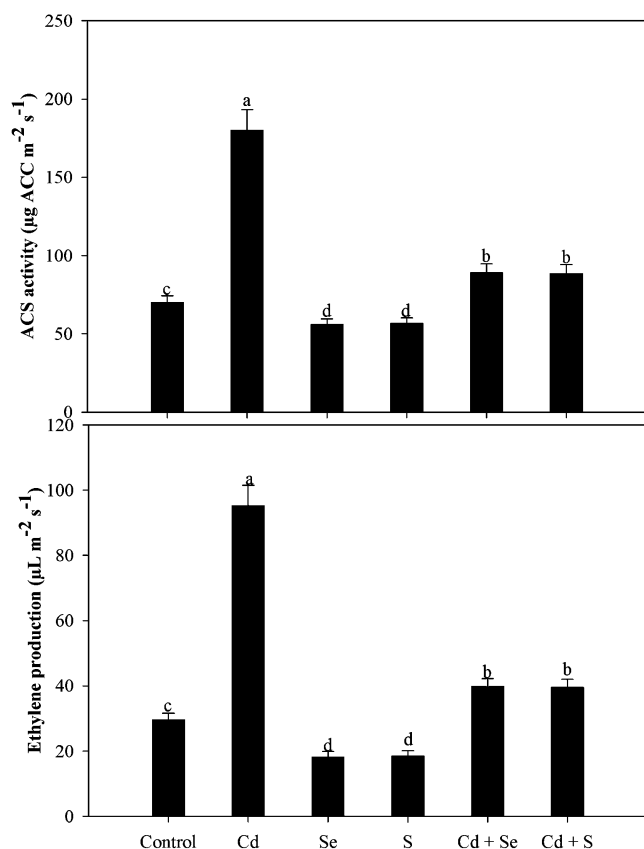


Fig. 6. ACS activity and ethylene production in wheat at 30 DAS. Plants were grown individually with 0, 200 mg Cd kg⁻¹ soil, 2 mg Se kg⁻¹ soil and 200 mg S kg⁻¹ soil or Cd combined with Se or S. Data are presented as treatments mean \pm SE ($n=4$). Data followed by same letter are not significantly different by LSD test at ($P<0.05$).

and function (Kavi-Kishor et al., 2005), regulates cellular redox potential (Ashraf and Foolad, 2007), sustains PSII electron transport (Hamilton and Heckathorn, 2001), increases N remobilization and N-use efficiency in *B. napus* (Albert et al., 2012). Independent studies on Se and S have shown that they improve photosynthetic efficiency and proline accumulation under salt stress in *B. juncea* and under chilling stress in *Cucumis sativus* (Hawrylak-Nowak et al., 2010; Rais et al., 2013).

The findings of this study suggest that chlorophyll fluorescence parameters were reduced under Cd-treated plants and contributed to the decrease in the net photosynthesis (Table 1). The decrease in photosynthetic efficiency (Φ PS II, F_v/F_m and F_v'/F_m'), ETR and qP with Cd treatment and increase in NPQ was observed (Table 2). Mallick and Mohn (2003) have suggested that the decrease in chlorophyll fluorescence under Cd stress results from destruction of antenna pigments by the partial block of electron transport from PSII to PSI. Reversal of Cd-induced photosynthetic inhibition was achieved by the application of Se and S equally. It is suggested that an increase in efficiency of PS II by Se and S treatments under Cd stress increased electron transport which helped plants to limit singlet oxygen production at PS II resulting in increased PSII activity under stress. Increased SPAD values in Cd-stressed plants after Se or S application reflected higher chlorophyll synthesis which contributed to increase in photosynthesis. Selenium can protect and increase chloroplast size under Cd stress and can partly counter-balance the destructive effects of Cd (Filek et al., 2010). Increased N assimilation in plants receiving Se and S provided N backbone for chlorophyll and increased tolerance of wheat plants to Cd stress. The other mechanisms operating with increased N metabolism after Se or S application to Cd-stressed plants were increased GSH

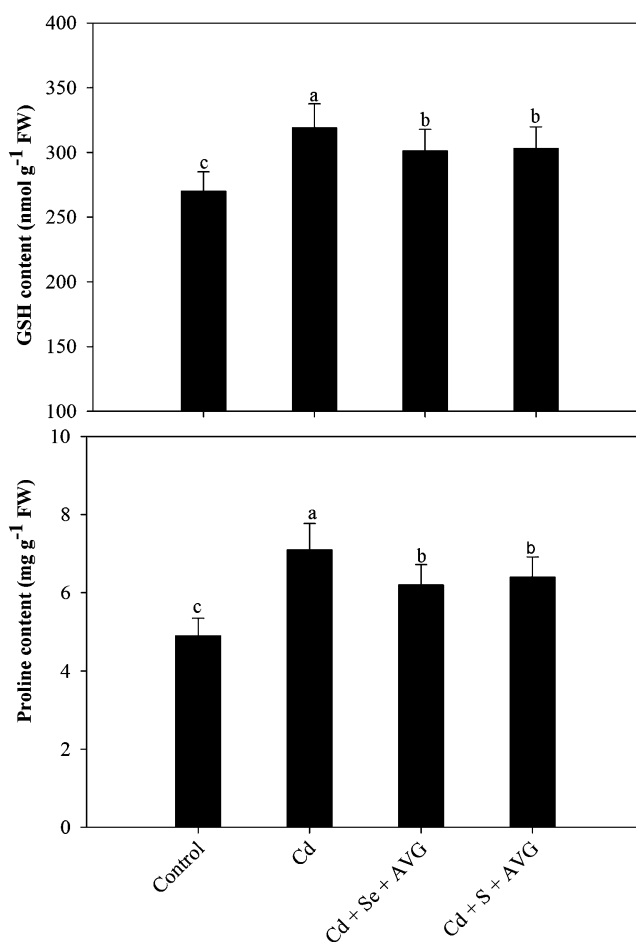


Fig. 7. Content of GSH and proline in wheat at 30 DAS. Plants were grown individually with 0, 200 mg Cd kg⁻¹ soil or combined treatment of 2 mg Se kg⁻¹ soil or 200 mg S kg⁻¹ soil with 50 μ L L⁻¹ AVG. Data are presented as treatments mean \pm SE ($n=4$). Data followed by same letter are not significantly different by LSD test at ($P<0.05$).

synthesis and ethylene formation. Earlier, it has been shown that ethylene increases photosynthesis via increase in the allocation of N and S to Rubisco in *B. juncea* and *T. aestivum* crops (Iqbal et al., 2012; Khan et al., 2013), but no report is available on the comparative effects of Se and S on photosynthesis under Cd stress involving antioxidant metabolism and ethylene formation in the alleviation of Cd-induced oxidative stress.

Supplementation of Se or S to Cd-treated plants increased ATP-S and SAT activity and GSH content together with lower values of H₂O₂ content and lipid peroxidation indicating the role of GR and GPX in GSH production and decomposition of H₂O₂ resulting in improved photosynthesis (Fig. 4). The protection of photosynthetic inhibition by oxidative stress was attributed to the lowering of ethylene level under Cd stress by Se or S application. Ethylene level in the optimal range regulates GSH synthesis and redox state of the cell. Involvement of ethylene in the regulation of GSH has been suggested in *Arabidopsis* under ozone stress (Yoshida et al., 2009), in *B. juncea* under metals (Cd, Ni and Zn) stress (Masood et al., 2012; Khan and Khan, 2014) and salt stress (Nazar et al., 2014) and *Vigna radiata* under salt stress (Khan et al., 2014a,b). Results with ethylene biosynthesis inhibitor AVG have also shown that inhibition of ethylene increased the inhibition of photosynthesis and growth by Cd and suggested that ethylene plays a role in the alleviation of Cd stress by Se or S (Fig. 8). Khan and Khan (2014) reported that ethylene application resulted in the reversal of photosynthetic inhibition by Ni and Zn stress.

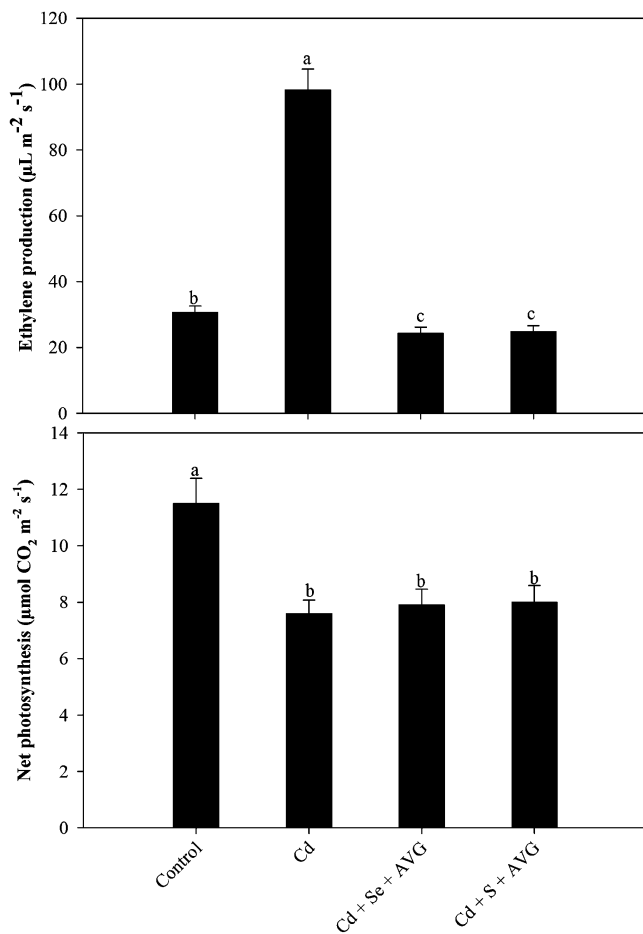


Fig. 8. Ethylene production and net photosynthesis in wheat at 30 DAS. Plants were grown individually with 0, 200 mg Cd kg⁻¹ soil or combined treatment of 2 mg Se kg⁻¹ soil or 200 mg S kg⁻¹ soil with 50 μL L⁻¹ AVG. Data are presented as treatments mean ± SE (n=4). Data followed by same letter are not significantly different by LSD test at (P<0.05).

In conclusion, it may be said that Se and S affect proline and antioxidant metabolism and photosynthetic processes under non stress and stress conditions. Application of Se or S equally promotes N and S assimilation, proline and antioxidant metabolism and ethylene formation in the optimal range under Cd stress. The optimal ethylene resulting from Se or S application under Cd stress regulates proline and GSH biosynthesis and alleviates adverse effects of Cd stress on photosynthesis and growth. The physiological response of wheat plants to Se and S application and ethylene-mediated proline and GSH production can be used in augmenting photosynthesis and growth under Cd stress. This may be exploited as a physiological tool in sustainable development of wheat plants.

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